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Microbial Biosensor for the Detection of Protease-Virulent Factors from Pathogens

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14. ABSTRACT
The PI has been successful in the detection of protease-virulent factors from pathogens through wholecell and protein-based biosensor designs. Other means of detecting pathogens are reported through the detection of microbial signaling molecules and environmental cue sensing. The PI designed a protease sensing whole-cell biosensor, and they compiled a library of tools used to engineer the microbial cell to respond at different threshold concentrations and response time. This confers the advantage of flexibility in detecting the protease-toxin which can pave the road for engineering microbes to facilitate bioremediation of contaminated water sources. The protein-based biosensor created functions as a quick method of detection that offers rapid response time. Used in tandem, both of these biosensors would function to detect the presence and clear the toxin. These biosensors demonstrated could be added on to the toolkit for detection of other contaminants that would be present in the water supply. As a whole, the design of these biosensors can provide rapid identification of various types of contaminants in a water supply and ultimately serve to reduce the possibility of water-related infections for deployed individuals.

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Microbial Biosensor for the Detection of Protease-Virulent Factors from Pathogens

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Abstract: The detection of protease-virulent factors from pathogens was achieved through whole-cell and protein-based biosensor designs. Other means of detecting pathogens were demonstrated through the detection of microbial signaling molecules and environmental cue sensing. To design a protease sensing whole-cell biosensor, we compiled a library of tools used to engineer the microbial cell to respond at different threshold concentrations and response time. This confers the advantage of flexibility in detecting the protease-toxin, and paves the road for engineering these microbes to facilitate bioremediation of contaminated water sources. The protein-based biosensor, on the other hand, functions as a quick method of detection that offers rapid response time. Used in tandem, both of these biosensors would function to detect the presence and clear the toxin. These biosensors demonstrated could be added on to the toolkit for detection of other contaminants that would be present in the water supply. As a whole, the design of these biosensors can provide rapid identification of various types of contaminants in a water supply and ultimately serve to reduce the possibility of water-related infections in on-field servicemen.

Introduction

Before the 1920s, pathogen infections pose a deadly threat to human lives. During the First World War, many of the injured soldiers lost their limbs and in certain cases their lives due to open wounds infected though exposure to unsanitary conditions. Noting that many of the microbes were not classified, Alexander Fleming cultured microbes found on the clothings and wounds from war patients and discovered that over 90.3% of the wounds infected carried similar microbial infection. It was not until 1928, when he discovered penicillin that resolved the issue of microbial infection at that time. Since then until the 1960s, the race to discover new antibiotics followed by their pervasive usage proved useful in combating various forms of infection. However, the overutilization and misuse of these antimicrobial agents led to the recent emergence of deadly antibiotic-resistant strains, such as MRSA (methicillin-resistant *Staphylococcus aureus*) and VRE (vancomycin-resistant *Enterococci*). Military personnel are often at risk of pathogen infection resulting from the job related-isolation and stressful conditions that may lead to long-term medical and psychological complications or fatality.

Waterborne microbes are usually the source of most infections and can occur from surface-surface interactions, washing, bathing and consumption of food or beverages prepared using contaminated water. This may lead to various illnesses such as respiratory infections, skin diseases, and gastrointestinal infections that were the commonly reported diagnoses during the U.S. military deployments in Iraq and Afghanistan. Approximately 77% and 54% of the U.S. military personnel deployed to Iraq and Afghanistan, respectively, suffered from diarrhoea, and 69% experienced respiratory infections.

Although immunization would be the preferred means of preventing an infection, detection of contaminated water sources could drastically reduce these risks. Additionally, though quick analysis of the water content, appropriate point-of-care could be provided on time, reducing the effects of infection risk factors, such as toxins. The fastest and most cost effective manner of detection is to

specifically bind and respond upon detection of infection-causing molecules such as toxins and pathogen signalling molecules. With that goal in mind, researchers have developed various types of biosensors that detect infectious determinants using antibodies, imprinted polymer, aptamers, and nanoparticles. Whole-cell biosensors are known for their versatility in identifying various target molecules such as heavy metals, antimicrobials, environmental pollutants and microbial pathogens. Whole-cell biosensors are genetically modified to respond to environmental stimuli and provide fluorescent, bioluminescent or colorimetric outputs that are further processed and interpreted by optical readers. Detectable biomolecules must be diffusible across cell membranes and importable into the cytoplasm to facilitate the biomolecular reaction. Alternatively, the biomolecules can directly interact with cell surface receptors to initiate a cascade of signal transduction that leads to the intracellular genetic expression of output reporters. Commonly studied signals include small molecule-based signalling mechanisms such as quorum sensing and virulence factors such as protease toxins. However, virulence factors are usually large proteins that are not membrane permeable thus limiting the sensing capacity of the engineered biosensory cells. To circumvent this limitation, we proposed, in this project, to rewire whole-cell microbial biosensors to sense extracellular virulence determinants, specifically protease toxins from pathogens using an artificial signalling cascade. One such approach was to **reconfigure native two-component signal transduction system (TCS) of microorganisms such that the engineered whole-cell biosensor will be responsive to protease toxins in extracellular space**. We further **expressed protein biosensors that are responsive to protease toxins**, as well as **other means of detecting the presence of pathogens**.

The project would contribute to the development of the **next-generation whole-cell biosensors** for toxin-based pathogen detection. Employing synthetic biology approaches, our various constructs will endow military personnel with the capability of identifying and controlling potential threats originating from pathogenic biological agents.

Experiment

Whole-cell protease biosensor construction

The objective of our study was to sense pathogen released-protease toxin in water supply. Our initial design took advantage of the native gram-positive microbial two-component signals transduction system (TCS) and we engineered the proteins to be responsive to the protease toxins cleavage in the extracellular space. The cleavage of TCS receptor protein would abolish the kinase activity responsible for the phosphorylation of the response regulator for the first promoter, thus disrupting the expression of a repressor peptide. This repressor peptide binds to a second promoter that upon disruption results in the restoration of the reporter protein expression **(Figure 1).**

Our first task was the generation of constructs and the screening of various transmembrane and promoter combination. The screening assay data was subsequently used for optimizing biosensor construction for signal sensitivity and shorter response time. Finally, the lyophilized whole-cell biosensor was immobilized on a membrane paper sandwich to form the paper-based biosensor. The

envisioned biosensor would enable users to detect the presence of pathogens in water samples with a colorimetric output.

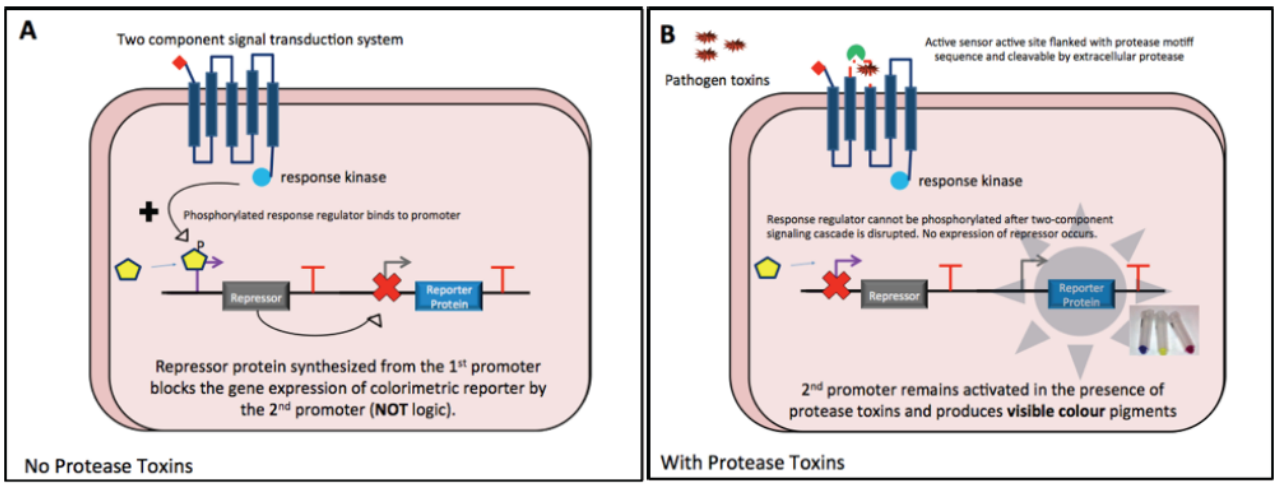

Figure 1| The two-component signals transduction (TCS) biosensor. The functional output of the TCS biosensor in the **(A)** absence and **(B)** presence of the target protease.

We selected the protease-deficient *Bacillus subtilis* WB800N as a candidate microbe because it is a gram-positive non-pathogenic microorganism with good survival and genetic expression traits in a non-laboratory environment. In addition, it is well characterized and have an accessible repertoire of genetic databases and microbiological protocols.

We adopted the well-studied gram-positive *Staphyloccocal* Agr quorum sensing system to study its transmembrane protein structure analysis and library generation (**Figure 2**). In this heterologous system, we engineered the host to produce and secrete autoinducing peptide (AIP) through the expression of AgrB and AgrD. The local concentration of AIP would trigger AgrC, a transmembrane receptor histidine kinase that phosphorylates a cytoplasmic response regulator, AgrA, which functions as a transcriptional activator.

As the cell-based protease biosensor response requires over an hour to have any detectable signal, alternative rapid detection designs were explored to complement the current biosensor design. The cell-based biosensor would be used as a means to engineer microbes for bioremediation purposes.

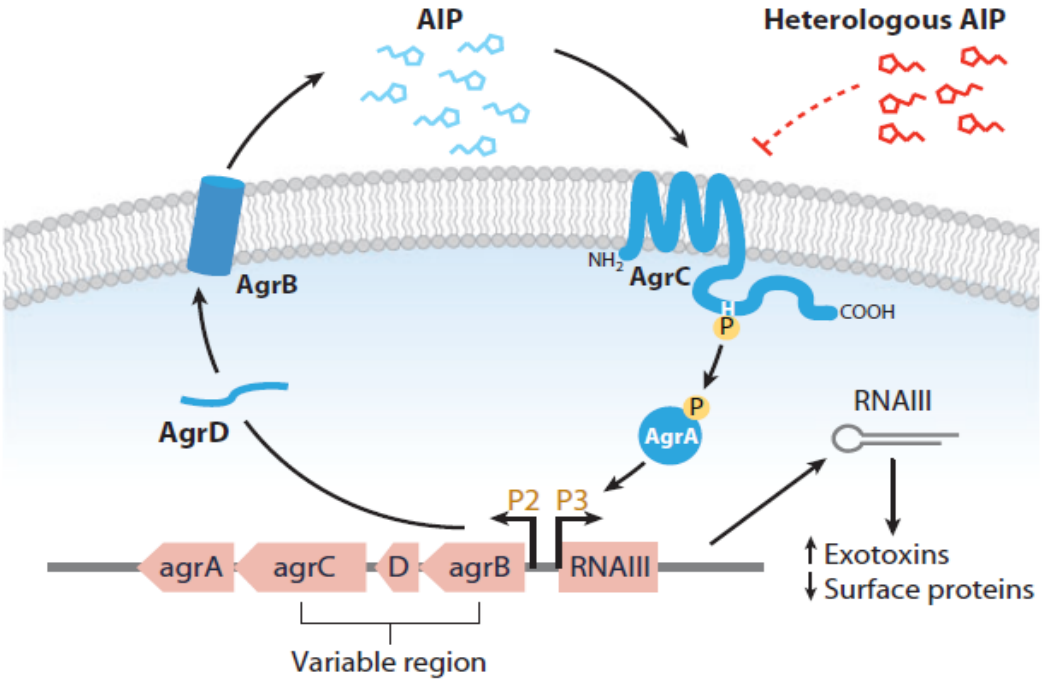

Figure 2 | The *Staphylococcal* Agr quorum sensing system. AgrD is a propeptide in producing AIP; AgrB is the transporter that modifies AgrD to AIP; AgrC is a AIP receptor that phosphorylates AgrA, an activator for P2 and P3.

Protein-based protease biosensor construction

To facilitate rapid detection of protease toxins, we designed a fusion protein that carries a GFP coupled to a peptide cleavage site that is linked to the GFP quencher peptide. By using the protein-based biosensor, immediate protease activity readout would enable rapid identification for contaminated water though fluorometric and colorimetric means. When GFP is fused to a quencher peptide derived from the tetrameric proton channel domain of influenza M2 protein, the fluorescence detected is minimal. This is a result of the steric clash between adjacent protein barrels during the tetrameric unit formation (**Figure 3**). With the addition of a protease cleavage site between the GFP and quencher peptide modules, the target protease will cleave at the specific recognition site, allowing GFP to fully mature and fluoresce strongly. In our pilot study, we investigated the design using a TEV cleavage site as a proof of concept. We are progressively testing other protease recognition sites such as Elastase B from *Pseudomonas aeruginosa* and Sortase A from *Staphylococcus aureus*.

Using this approach, it is possible to detect the levels of protease toxin in a water sample using fluorescence spectrometry or through colorimetric changes in the samples.

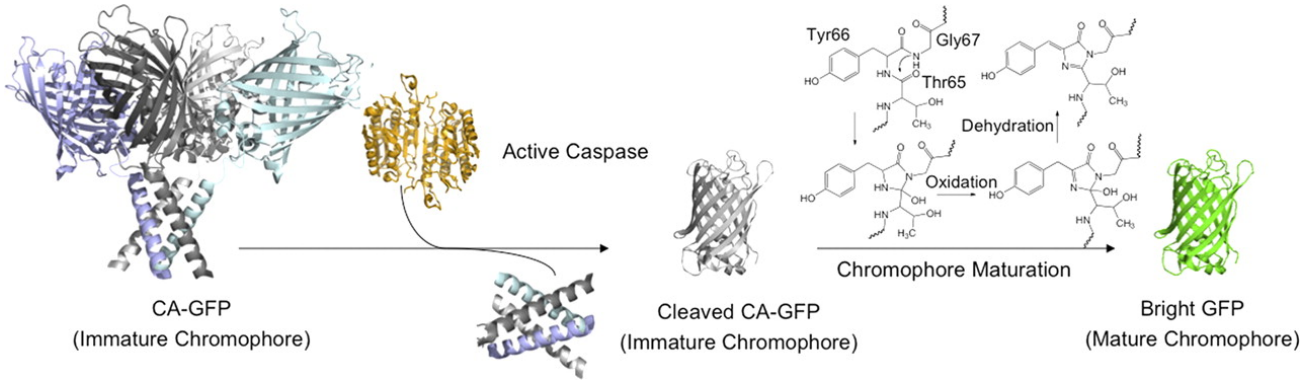

Figure 3| The CA-GFP quenching mechanism that is the basis of the protein-based biosensor. The quencher peptide forms a homotetramer structure that prevents the maturation of the GFP chromophore. Upon protease cleavage, the freed GFP folds normally thus restoring the hydrophobic barrel for GFP chromophore maturation.

Biosensor design for detecting inter-cellular signals and environmental cues

Based on a similar system, we further developed whole cell biosensors that intercepts intercellular communication signals from both gram-positive and gram-negative bacteria. Gram-positive bacteria send signals by the means of autoinducing peptides (AIP) and are vastly found in communities in the *Staphylococcus* genera. Gram-negative microbes, such as those from the *Vibrio, Yersinia* and *Pseudomonas* genera, utilize quorum sensing molecules such as AI (autoinducer) I, II and III to communicate (**Figure 4**). The changes in the concentrations of these autoinducers would result in various changes in the recipient cellular biochemical response to the signal. By piggy-backing on these pre-existing receptor proteins or introducing foreign genes expressing these receptor proteins, we have engineered various cells that respond by producing detectable signals that would report the presence of the pathogen cells. Additionally, the design biosensor would function as a suitable tool for high throughput screening of potential antibiotics or virulence repressors that can be used to target pathogens.

It is known that some pathogenic microorganisms are capable of modulating environmental pH by secreting acids or alkali. A biosensing system is needed to report the pH changes made by such pathogens. To advance the tools needed to evaluate the water quality on field, we further devised the biosensor to detect changes in pH using riboswitches as a form of pH biosensor.

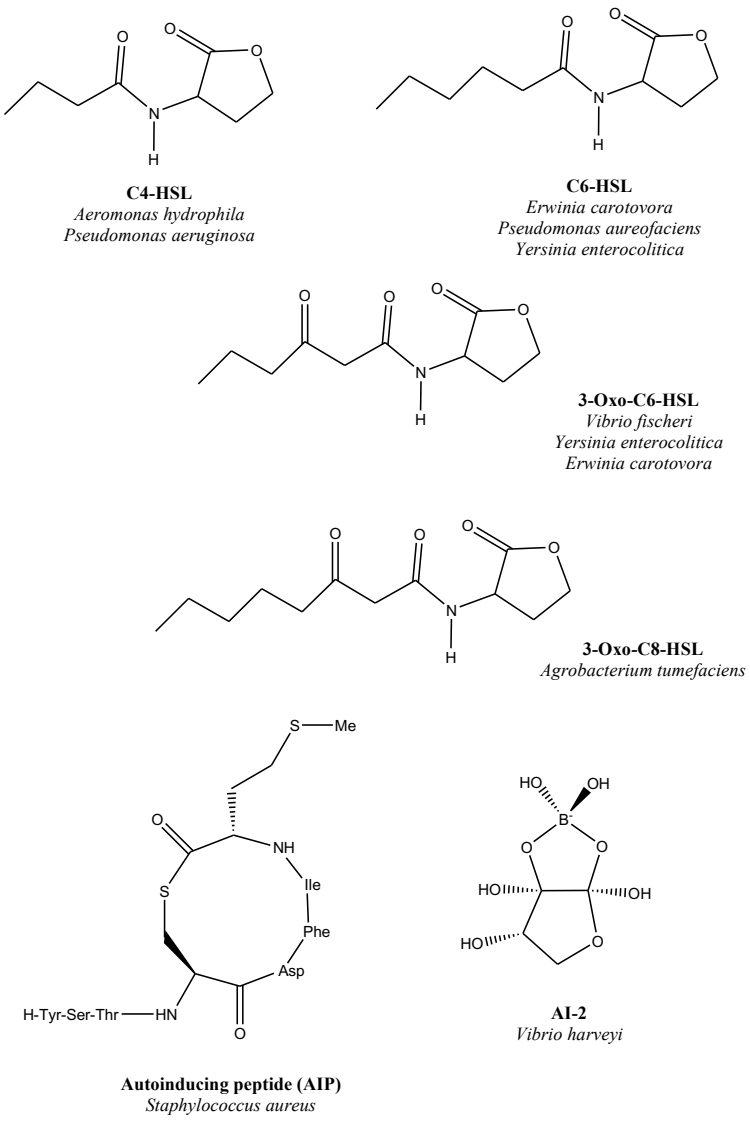

Figure 4| Various quorum sensing molecules and their producers

Results and Discussion

Whole-cell protease biosensor construction

We compiled a library of various AIP detecting systems that were designed based on AgrC and AgrA (**Figure 5, Table 1**) with superfolder GFP(sGFP) as the reporter. The expression of AgrA and AgrC was driven by the IPTG-inducible Pgrac promoter in *B. subtilis* WB800N. For the AIP detecting systems, expression of the reporter sGFP was driven by either -phosphorylated-AgrA-regulated promoters, P2 or P3. Based on the hydropathy plot and TMHMM prediction, AgrC could be divided into 6 transmembrane regions with 3 extracellular and 4 intracellular regions (**Figure 6**).

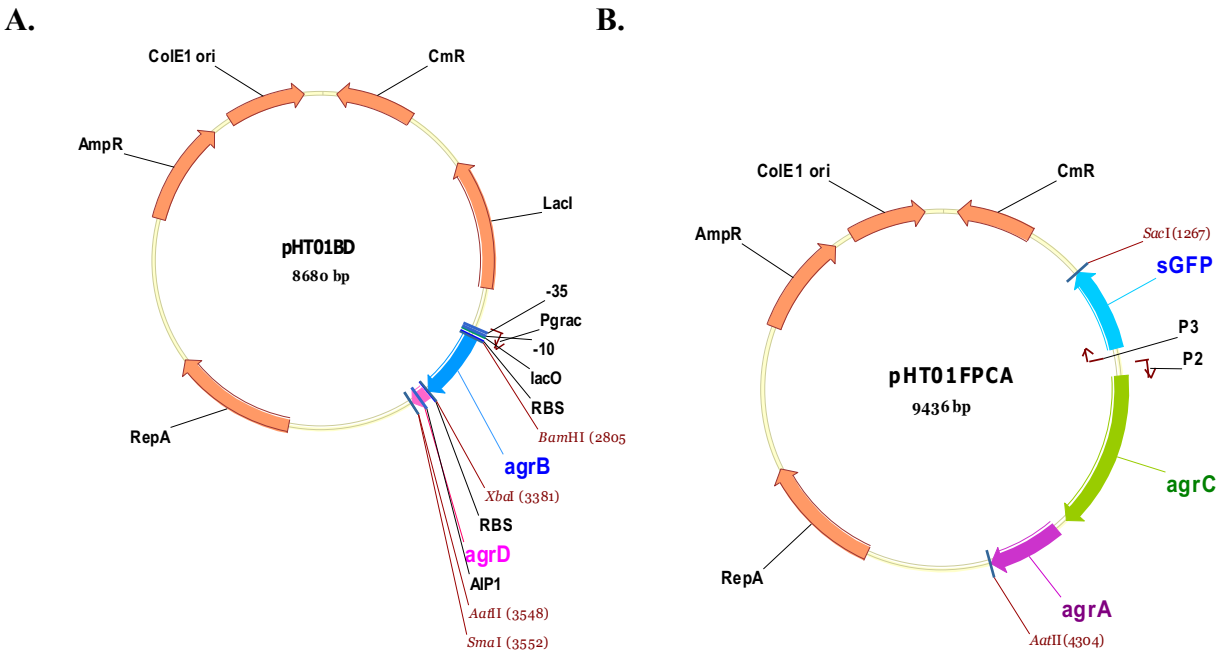

Figure 5| A. pHT01-based AIP generating system. **B.** AIP detecting system.

Table 1| Sensing systems constructed in this study.

Sensing system	Characteristics
Agr system	
pHT01-FPCA	P2-AgrC-AgrA; P3-sGFP
pHT01-FPC(m)A	P2-mutantAgrC-AgrA; P3-sGFP
pHT01-FP2AC	P_{grac}-AgrA-AgrC; P2-sGFP
pHT01-FP2AC(m)	P_{grac}-AgrA-mutantAgrC; P2-sGFP
pHT01-FP3AC	P_{grac}-AgrA-AgrC; P3-sGFP
pHT01-FP3AC(m)	P_{grac}-AgrA-mutantAgrC; P3-sGFP
pHT01-FP3ACS	P_{grac}-AgrA-AgrC-SarA; P3-sGFP
SURE system	
pHT01-FRK	P_{grac}-SpaR-SpaK; P_{SpaS}-sGFP
pHT01-FRK(m1)	P_{grac}-SpaR-mutantSpaK1; P_{SpaS}-sGFP
pHT01-FRK(m2)	P_{grac}-SpaR-mutantSpaK2; P_{SpaS}-sGFP

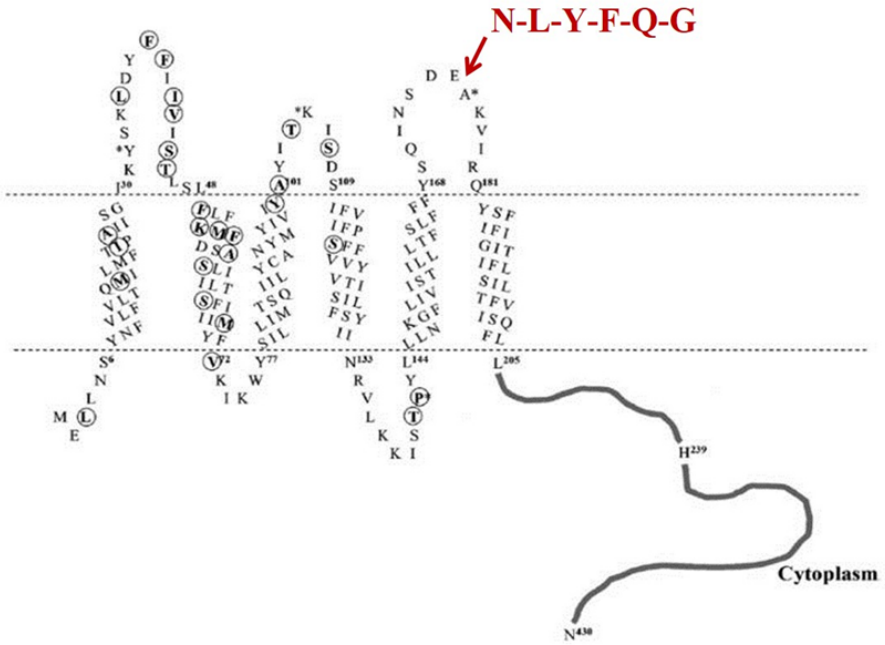

Figure 6 | Insertion of TEV cleavage site on AgrC

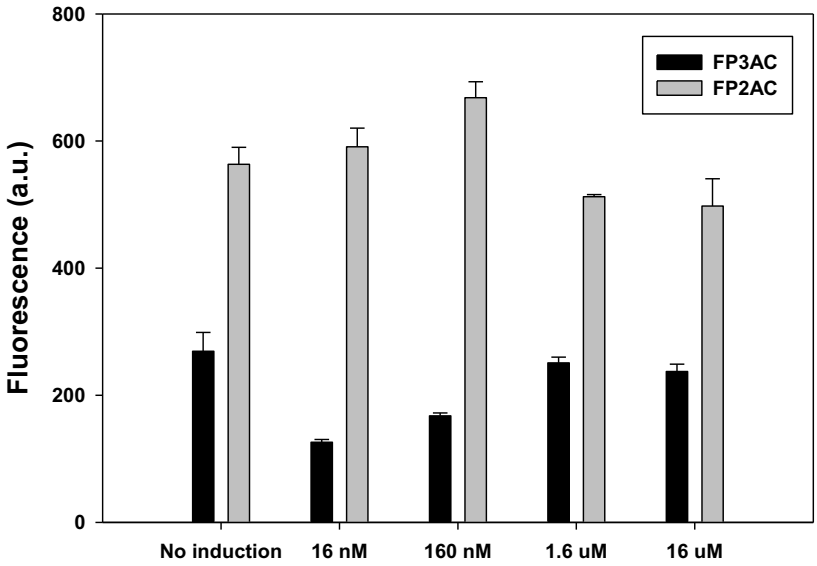

Figure 7| The Agr system-based biosensor response to varying concentrations of synthetic AIP. *(FP2AC: The B. subtilis with pHT01-FP2AC; FP3AC: The B. subtilis with pHT01-FP3AC).*

We engineered the TEV protease cut site at the third extracellular loop of AgrC (**Figure 6**). This is because the first and second extracellular loops play a role in receptor activation and AIP recognition. Based on structural analysis, we inserted the TEV cleavage site (NLYFQG) between E175 and A176 (**Figure 6**). This is due to the folding of the loop, where the exposed side chain residues would favor cleavage by the protease. By incorporating this AgrC construct to the host cell with the functional AgrA, we designed a cell-based biosensor that expressed the reporter protein in the presence of TEV protease. To ensure our construct was fully functional, we tested our library of constructs with *B.*

subtilis generated AIP, *S. aureus* supernatant of culture, and commercially synthesized AIP. We observed that there was no significant difference between the uninduced and the induced state. However, we did observe a consistent repression within the nanomolar range (**Figure 7**). We are currently in progress of optimizing the sensory domain and the promoter to improve responsiveness to protease toxins.

We have also begun testing the subtilin regulated expression system (SURE) derived from *Bacillus* as an alternative to the *Staphylococcal* Agr system. Based on the hydropathy plot and TMHMM prediction, there were two transmembrane domains in SURE receptor protein, SpaK (**Figure 8**). We are currently evaluating the functionality of this receptor domain for its use as a protease toxin biosensor.

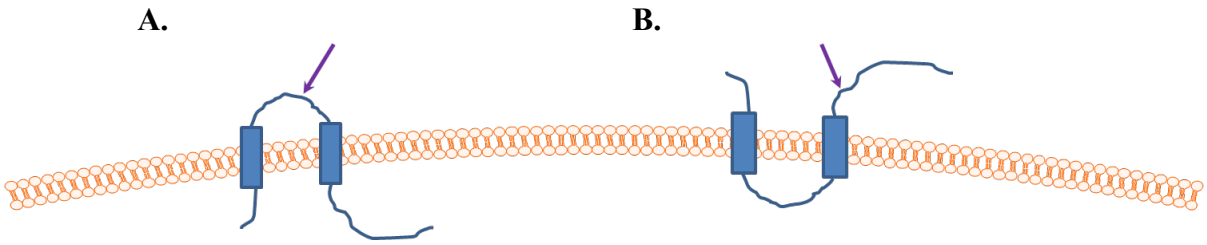

Figure 8| Two possible topology for SpaK, in which the center loop is possibly located at the (**A**) extracellular milieu or (**B**) intracellular matrix. Arrows indicate location proposed TEV protease recognition sequences.

This study would impact how gram-positive microbes can be engineered to function as biosensors and the knowledge of programming cells would be useful for therapeutic and environment-based applications. Engineering of whole-cell biosensors for detection of protease toxins would be particularly useful in monitoring water supply where its application could be extended to the bioremediation of pathogen-contaminated water supplies.

Protein-based protease biosensor construction

In the design of peptide-based protease biosensor, we discovered that varying the number of glycine-serine linker between the protease cleavage site and the quencher peptide resulted in improved change in quencher peptide release upon cleavage. Based on our observation, we noted that the addition of linker to the quencher peptide results in lower background signal (**Figure 9 and 10**). This could be caused by the arrangement of the quencher peptide during the formation of the homotetramer. The other possible reason is that the TEV protease recognition sequence (ENLYFQC) maybe be interfering with the orientation of the GFP barrel resulting in GFP maturation prior to TEV cleavage. It was noted that the increased number of GS linker to 25 or 30 amino acids reduced the fold difference compared to GFP-TEV-20-QP and QP-25-TEV-GFP. The shorter linker might influence the exposure of the TEV site for effective cleavage by the protease, thus limiting the amount of matured GFP expression. We further noted that the arrangement of having the GFP expressed at the C-termini of the protein sensor gave a 78-fold increase compared to 11-fold in the N-termini GFP variant (**Figure 9**).

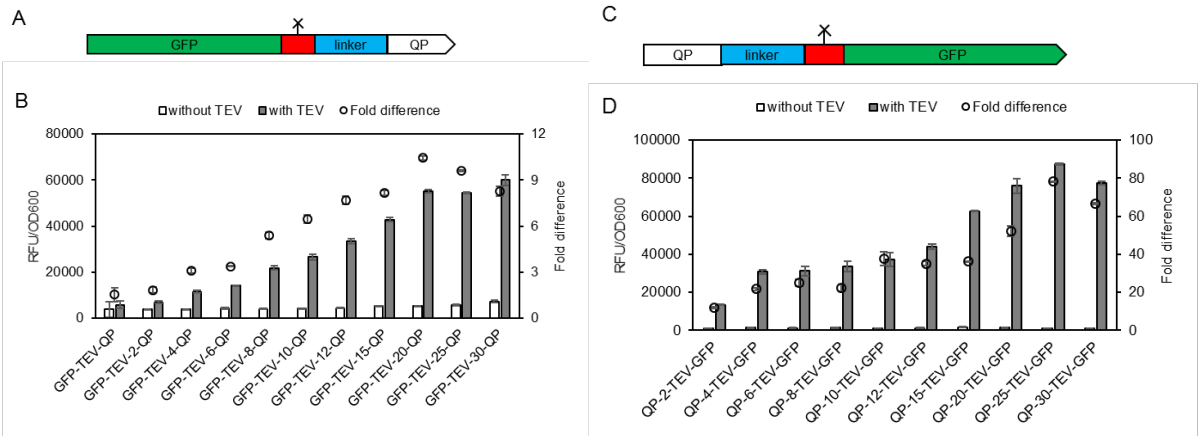

Figure 9| Insertion of GS linker domains between TEV cleavage site and the quencher peptide. The N-terminus GFP construct (**A**) and the biosensor with varying GS repeats *(indicated by the number)* response to TEV cleavage (**B**). The C-terminus GFP construct (**C**) and the biosensor with varying GS repeats *(indicated by the number)* response to TEV cleavage (**D**).

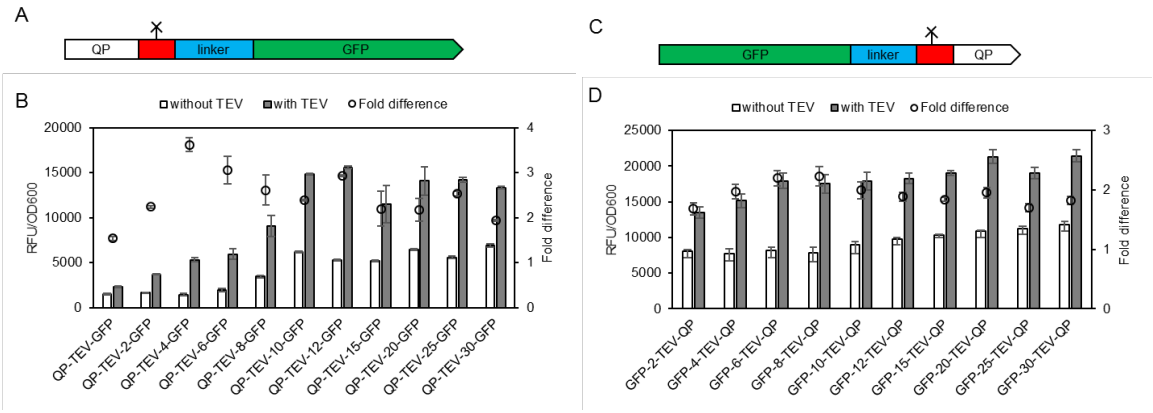

Figure 10| Insertion of GS linker domains between GFP and the TEV cleavage site. The N-terminus GFP construct (**A**) and the biosensor with varying GS repeats *(indicated by the number)* response to TEV cleavage (**B**). The C-terminus GFP construct (**C**) and the biosensor with varying GS repeats *(indicated by the number)* response to TEV cleavage (**D**).

Currently, we are in the process of investigating on the various combination of linkers located on both the cleavage site ends. Studies on the protein stability for immobilization on a paper based matrix are ongoing. We believe that this sensor when stable would be able to function as both a rapid sensor to determine the contaminated water and as a high-throughput manner of drug screening against pathogens. Little has been studied on the correlation of the linker domain and how it effects the maturation of the GFP barrels. Thus, advances in this study would shed light on how protein biosensors that detect proteases could be further enhanced to produce a more sensitive sensor with higher changes in reporter signals.

<u>Biosensor design for detecting inter-cellular signals and environmental cues</u>

In the effort to detect quorum sensing signals from gram-positive pathogens, we studied extensively on using the Agr system to detect the presence of AIP. By arranging the expression alignments of

AgrA, AgrC and the glucuronidase reporter for enzymatic detection, we were able to design two variants of AIP biosensors that detect AIP concentrations at sub-nanomolar and nanomolar concentrations. The first version of the sensor was capable of detecting at nanomolar concentrations, while the improved version 2 measured concentrations within the subnanomolar range, as shown in the kinetic assays performed at varying concentrations of AIP (**Figure 11A and 11B**). The readout was consistent with the expression of glucuronidase enzyme by the cells in the presence of different AIP concentrations (**Figure 11C**). By using both sensors in tandem, it is possible to determine the concentrations of AIP in a solution. The sensors could also be used as a high throughput assay for determining the AIP threshold to elicit a response from the recipient cells.

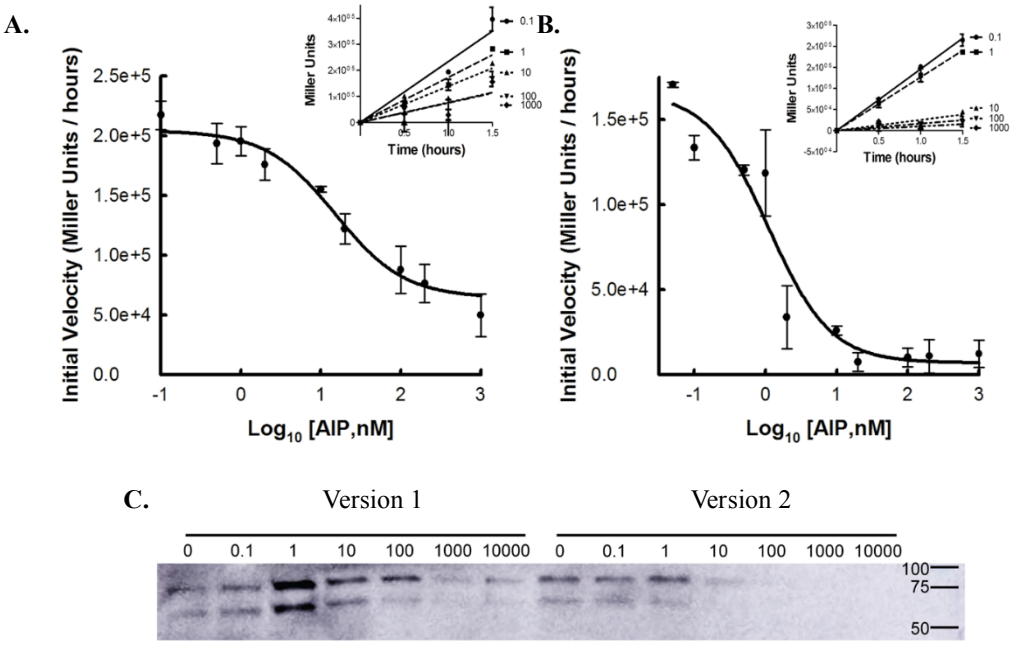

Figure 11| Kinetic assay of (**A**) sensor version 1 and (**B**) sensor version 2. (**C**) Glucuronidase expression levels using sensors version 1 and 2 under different AIP concentrations.

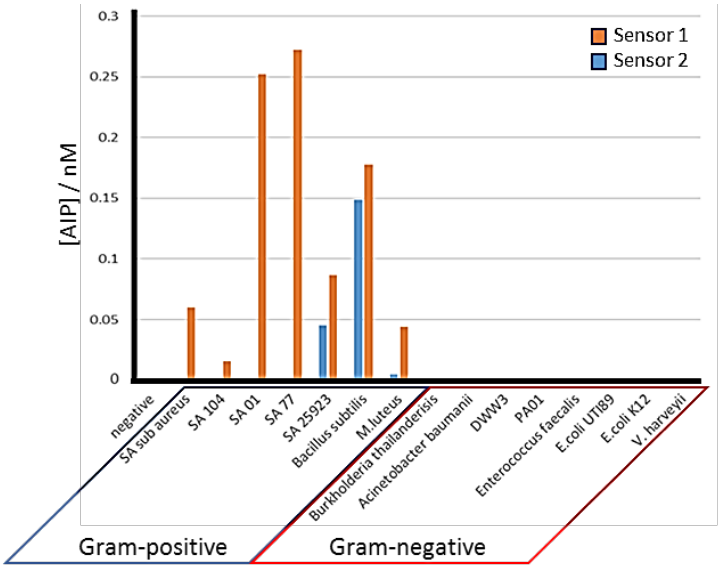

Figure 12| Sensor detection of AIP levels produced by various microbes during early exponential phase.

We tested our biosensor for the detection of AIP release from various microbes and it was found to be sensitive to only gram-positive microbes producing autoinducing peptides (**Figure 12**). This design could thus detect the presence of gram-positive microbes and be used in a high throughput manner for detecting AIP produced under different environment conditions.

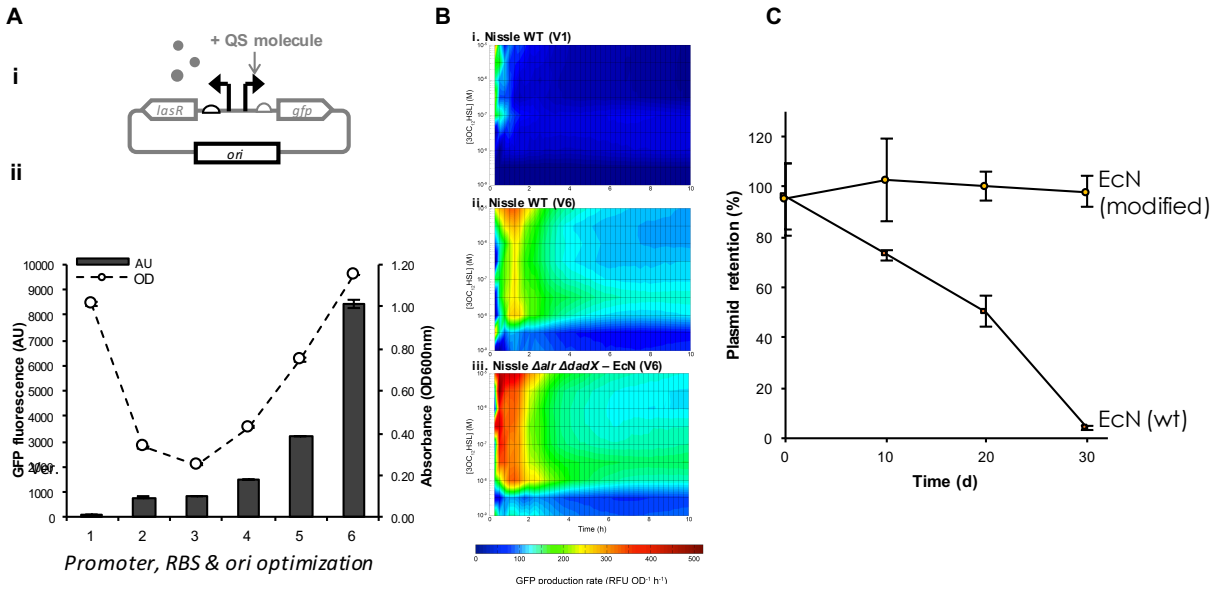

Figure 13| Development of optimal cellular host and genetic system for functional probiotic strain. **A**. Optimization of the sensing system. (i) Several variations in the regulatory components (highlighted in black in the circuit diagram) was performed. (ii) Level of activation by the variations was reflected by GFP expression and bacterial growth. **B**. Lower threshold for pathogen detection via QS molecule (e.g. AHL) and sustained expression was achieved in the new vector-host system as a functional probiotic strain. **C**. Auxotrophic complementation was applied to bypass antibiotic-dependent expression and stabilize plasmid retention. As a result, the extent of plasmid retention and expression from the vector in the absence of antibiotic (selection pressure required for plasmid retention by the cells) are superior in the modified EcN strain.

In the design of gram-negative pathogen signals, we based it on initial work published in 2011. Although the threshold of our reported sensing device was within the range of quorum sensing (QS) molecule secreted by the pathogen, we aimed to further optimize the genetic device for increased sensitivity for biosensing purposes. To this end, the genetic circuit, which controls the expression of sensing devices such as promoter, ribosome binding sites and replicon (**Figure 13A**), was optimized to achieve a 10-fold increase in sensitivity (**Figure 13B**). Furthermore, to ensure genetic stability of the sensing device in *E. coli* Nissle cells (EcN), strain engineering was performed to improve retention of the engineered plasmid. In *E. coli*, an introduced plasmid is not maintained in the absence of a selection pressure that reinforces the cells to retain additional DNA molecules. Typically, an antibiotic resistance gene is used for this pressure, thereby requiring costly antibiotics to be used. Therefore, auxotrophic complementation was applied where the functional gene was deleted in EcN and subsequently supplemented by the plasmid. Viability of the cell was dependent on retention of plasmid for complementation, thus securing plasmid stability (**Figure 13C**). A stable whole-cell biosensing system was developed by combining optimized sensing device with engineered cells for stable plasmid retention. This design coupled to the earlier gram-positive sensing design would facilitate the detection of various bacterial pathogens leading to better screening of sanitary water supply.

As part of the effort to design a pH sensor for detection of acidic water, we developed a riboswitch-based genetic platform for self-directed evolution acid-tolerant phenotype (RIDE) that is based on the flipping of the genetic construct governed by the riboswitch PREmR34. This riboswitch is sensitive to low pH conditions, and upon exposure to approximately pH5.0, the folded riboswitch would unravel turning on the expression of integrase 2 that facilitates rapid DNA switching (2~4 hours). Under normal pH conditions, the system would express dnaQ mutant that reduces cellular mismatch repair capacity, thus improving the sensor's sensitivity. Under acidic conditions, the expression of integrase 2 would facilitate the flipping of the RiboJ sites thus restoring the expression of RFP under the constitutive J23119 promoter. The robust response of the biosensor makes it a suitable tool for detecting low pH and could further be used to screen for mutants that would thrive in acidic conditions.

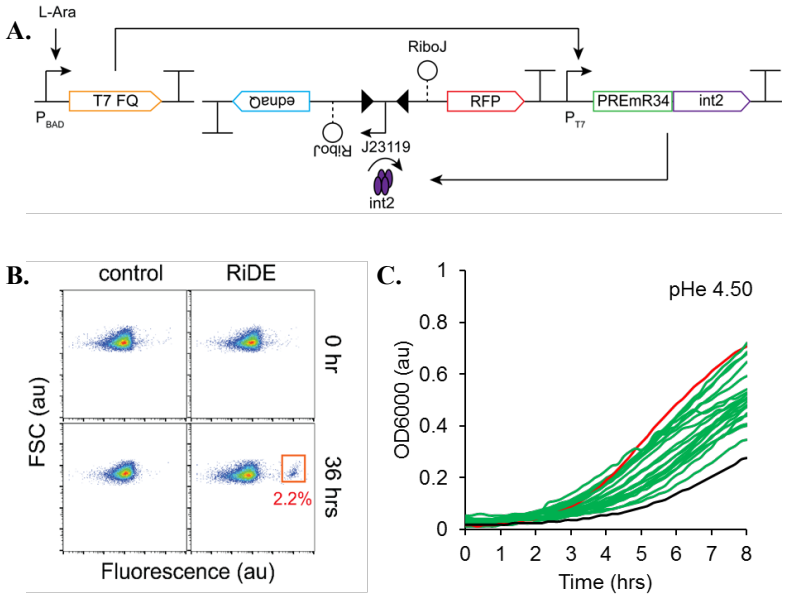

Figure 14| The riboswitch-based genetic platform for self-directed evolution acid-tolerant phenotype (RIDE) construct. **A**. Genetic circuit for RIDE (PREmR34: the pH sensitive riboswitch; int2: integrase 2; ednaQ: dnaQ mutant; RFP: red fluorescent protein) **B**. Sorted *E. coli* cells that were cultured in acidic conditions and the populations that are susceptible to growth. **C**. Growth of isolated cells that were acid-tolerant (red) against wild-type (black).

In summary, we have taken two approaches to detect protease-toxin in pathogen infested water. We further advanced our studies in sensing the presence of gram-positive and gram-negative microbes by detecting the secreted quorum sensing molecules produced by these microbes during pathogenesis. In addition, we developed a robust system to rapidly detect pH changes. The various biosensors designed show high sensitivity and can rapidly report the contaminant levels in water sources within a short span of time. The current models could be further optimized for better portability and increased shelf-life to boost their capability for rapid determination of clean water sources during field deployment. Despite the promising results of these studies, continued optimization is warranted before these tools can be employed as rapid water diagnostic kits. We believe that the completed sensor would help in determining clean water supply and could be further developed for detection of other poisons or toxins.

List of Publications and Significant Collaborations that resulted from your AOARD supported project

a) papers published in peer-reviewed journals

#Status	Published
Author Names	Jee Loon Foo, Hua Ling, Yung Seng Lee, Matthew Wook Chang
Title of Article	Microbiome engineering: Current applications and its future
Journal Name	Biotechnology Journal
Publisher	Wiley-VCH Verlag GmbH & Co. KGaA
Year/Issue/No.	2017/12/1600099
Page No.	1-9
Impact Factor	3.781
Date	2017/01/30

#Status	Published
Author Names	In Young Hwang, Elvin Koh, Hye Rim Kim, Wen Shan Yew, Matthew Wook Chang
Title of Article	Reprogrammable microbial cell-based therapeutics against antibiotic-resistant bacteria
Journal Name	Drug Resistance Updates
Publisher	Elsevier Inc.
Year/Issue/No.	2016/27
Page No.	59-71
Impact Factor	10.471
Date	2016/06/22

b) Papers published in non-peer-reviewed journals or in conference proceedings

N.A.

c) Conference presentations

N.A.

d) Manuscripts submitted but not yet published

#Status	In Press
Author Names	In Young Hwang, Elvin Koh, Adison Wong, John C. March, William E. Bentley, Yung Seng Lee, Matthew Wook Chang
Title of Article	Engineered probiotic Escherichia coli can eliminate and prevent Pseudomonas aeruginosa gut infection in animal models
Journal Name	Nature Communications
Publisher	Nature Publishing Group

Year/Issue/No.	2017
Page No.	Not applicable
Impact Factor	12.001

#Status	In press
Author Names	Hua Ling, Jee Loon Foo, Gourvendu Saxena, Sanjay Swarup, Matthew Wook Chang
Title of Article	Drug targeting of the human microbiome
Journal Name	Systems Biology
Publisher	Wiley-VCH Verlag GmbH & Co. KGaA
Year/Issue/No.	2017
Page No.	Not applicable
Impact Factor	Not applicable

#Status	Submitted
Author Names	Hoang Long Pham, Adison Wong, Niying Chua, Wei Suong Teo and Matthew Wook Chang
Title of Article	Engineering a riboswitch-based genetic platform for the self-directed evolution of acid-tolerant phenotypes

e) Interactions with industry or with Air Force Research Laboratory scientists or significant collaborations that resulted from this work

Two events have been resulted from this work:

1) Dr. Jermont Chen from AOARD visited NUS Synthetic Biology for Clinical and Technological Innovation (SynCTI) 21-22 Feb 2017
2) A visit by Dr. Matthew Chang from SynCTI to meet scientists of US Army has been scheduled to April of 2017.